BIODIVERSITAS
Volume 24, Number 12, December 2023
Pages: 6454-6460

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d241207

Molecular detection of iron gene on multidrug resistant avian fecal *Escherichia coli* isolated from broiler on traditional markets, Surabaya, Indonesia

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Manuscript received: 12 November 2023. Revision accepted: 14 December 2023.

Abstract. *Putri MFR, Kendek IA, Wibisono FJ, Effendi MH, Rahardjo D, Tyasningsih W, Ugbo EN. 2023. Molecular detection of iron gene on multidrug resistant avian fecal* Escherichia coli *isolated from broiler on traditional markets, Surabaya, Indonesia. Biodiversitas 24: 6454-6460.* Avian Fecal *Escherichia coli* (AFEC) is the cause of colibacillosis, which often infects the poultry industry throughout the world. The virulence gene influences the iroN factor, which causes systemic infections in poultry. This research aimed to determine Multidrug Resistance (MDR) in AFEC obtained from traditional markets in Surabaya. There were 96 cloacal swab samples from broiler chickens. The samples were isolated and identified using culture media in the form of Eosin Methylene Blue Agar (EMBA), gram stain, Triple Sugar Iron Agar (TSIA), Sulfide Indole Motility (SIM), Simmons Citrate Agar (SCA), Voges Proskauer (MR-VP) indole and methyl test; detection of multidrug resistance using Mueller-Hinton Agar (MHA) media. The results of the isolation and identification of *Escherichia coli* bacteria in this study showed that 60.4% of the isolates were positive for *Escherichia coli*. *Escherichia coli* is resistant to tetracycline antibiotics by 56%, ciprofloxacin by 55%, antibiotics aztreonam 29%, kanamycin 20%, and chloramphenicol 18%. The multidrug resistance test result on *Escherichia coli* was 25.8%. The PCR test results for the iroN gene were 40%. Therefore, there are MDR and iroN genes in avian fecal *Escherichia coli* in Surabaya traditional markets; APEC with iroN gene poses the potential to affect public health.

Keywords: Avian fecal *Escherichia coli*, iroN gene, broiler, colibacillosis, multidrug resistance, public health

INTRODUCTION

Escherichia coli is a bacteria that can be pathogenic (Aleksandrowicz et al. 2021). Colibacillosis is a disease caused by Avian Pathogenic *Escherichia coli* (APEC) (Alber et al. 2019; Johnson et al. 2006). This disease causes large economic losses in the poultry industry worldwide due to high morbidity and mortality rates (Tohmaz et al. 2022; Yu et al. 2020). Avian Pathogenic *Escherichia coli* (APEC) strains are one of the subgroups of Extraintestinal Pathogenic *Escherichia coli* (ExPEC) strains in chickens, ducks, birds, and other poultry species (Papouskova et al. 2020; Yu et al. 2020). APEC strains cause respiratory and systemic diseases in poultry, such as enteritis, septicemia, airsacculitis, perihepatitis, and pericarditis (Saha et al. 2020; Zhang et al. 2021). APEC has the potential to be a reservoir of virulence and resistance genes in human ExPEC, and virulence and resistance genes can be transmitted to humans with zoonotic potential (Filho et al. 2015; Hu et al. 2022). ExPEC in poultry and humans is related to several phylogenetic groups and the same genes with virulence, which may be the reason and require great attention to the zoonotic risk of APEC diseases (Wibisono et al. 2022). ExPEC diseases that attack humans mostly involve ExPEC strains that are resistant to antimicrobials; these strains have the special ability to cause disease in human internal organs (Yu et al. 2020). and infections of other extraintestinal organs, especially ExPEC, which has antimicrobial resistance and is transmitted through contaminated food (Hu et al. 2022). New problems related to the transmission of ExPEC from food, especially those originating from poultry products, have given rise to the emergence of diseases, including multidrug resistance, namely resistance to several antibiotics, so it is feared that APEC could cause economic problems and animal and human health problems (Filho et al. 2015).

APEC strains are influenced by several virulence factors, such as surface antigen, fimbriae, intimin, colicin, hemagglutinin, iron acquisition, serum resistance, and others (Tohmaz et al. 2022; Yu et al. 2020). APEC

virulence factors are controlled by several genes, including cvaC, tsh, sitA, iutA (aerobactin), ompT (outer membrane protease), hlyF (toxin), iss (serum resistance), and iron (salmochelin) which are found on the CoIV plasmid (Ramaditya et al. 2019; Tohmaz et al. 2022). The iron acquisition can affect the growth and proliferation of APEC in the host, such as aerobactin, salmochelin, and yersiniavaxin, and transport to iron absorbers in the body (Hu et al. 2022; Johnson et al. 2006). Siderophores are secondary metabolites that capture iron to increase bacterial growth and development. The iroN gene entering the blood serum can cause sepsis and infection in various organs that lack iron (Su et al. 2016). Several reports of APEC incidence influenced by various iroN virulence genes are 100% in Bangladesh (Hossain et al. 2021), Korea 100% (Jeong et al. 2021), 97% in Qatar (Johar et al. 2021), 92% in China (Subedi et al. 2018) and Indonesia 100% (Ramaditya et al. 2019) in broiler chickens.

Information about the spread of disease, phylogenetic relationships, and genes related to the emergence of APEC disease in Indonesia is still poorly explained and is generally a secondary disease, such as swollen head syndrome, chronic respiratory disease, and Newcastle disease (Prihtiyantoro et al. 2019; Wibisono et al. 2022). Therefore, antibiotic therapy in infected livestock is important to maintain livestock health and productivity (Kim et al. 2020). However, increasing antibiotic resistance continues to be reported in bacteria isolated from poultry (Li et al. 2014). Antibiotics that continue to experience resistance will trigger the emergence of multidrug resistance (MDR), where bacteria experience resistance to three or more different classes of antibiotics (Wibisono et al. 2020). Therefore, it is necessary to carry out this research to determine the incidence of multidrug resistance in AFEC related to APEC on iron genes in Indonesia.

MATERIAL AND METHODS

Ethical approval

Animal ethical approval was obtained from the Ethical Clearance Committee of the Faculty of Veterinary Medicine, Universitas Wijaya Kusuma Surabaya, Indonesia (ethics no.: 109D-KKE/2023).

Sample collection

The samples used were 96 cloacal swabs from broiler chickens in the traditional markets of Dukuh Kupang, Keputran, Pacar Keling and Pucang, Surabaya. The cloaca of broiler chickens was swabbed using a sterile cotton swab (Onemed, Indonesia). During transportation, all samples were placed in test tubes containing sterile buffered peptone water (HiMedia). All samples were transported using a thermobox at a temperature of 4°C (Yanestria et al. 2022).

Isolation and identification of *Escherichia coli*

Escherichia coli bacteria were isolated using Mac Conkey Agar (MCA) media (HIMEDIA MH081) and incubated at 37°C for 18-24 hours (Yanestria et al. 2022). Identification of *Escherichia coli* using gram staining and the biochemical tests IMVIC (Indol-motility, methyl red, voges proskauer) and TSIA (Triple Sugar Iron Agar) (Effendi et al. 2021; Wibisono et al. 2021).

Antibiotic sensitivity test

The sensitivity test used the disc diffusion method (diffusion test) isolated on Mueller-Hinton Agar (MHA) media (HiMedia M173) adapted from the Clinical Laboratory Standards Institute (CLSI 2018). The antibiotic disk (Oxoid, UK) used in this study is a group of antibiotics, including Aztreonam (monobactam), Chloramphenicol (phenicol), Kanamycin (aminoglycoside), Ciprofloxacin (Fluoroquinolone) and Tetracycline (Tetracycline). One to two colonies were taken from EMBA media using a sterile tube, then placed in physiological NaCl with Mc Farland standard 0.5 (1.5x108 CFU/ml). Then, rub gently with a sterile swab over the entire surface of the Mueller Hinton Agar (MHA) media. Incubate at 35°C for 16-18 hours under aerobic conditions. Interpret results by adjusting the diameter of the inhibition zone based on CLSI (CLSI 2020; Yanestria et al. 2022). Isolates are declared Multidrug-resistant (MDR), where bacteria are resistant to three or more different classes of antibiotics (Wibisono et al. 2020), as shown in Figure 1.

Characteristics of the iroN Gene using PCR

DNA extraction using QIAamp® DNA kit (QIAGEN, Germany). The forward primer used is AAGTCAAAGCAGGGGTTGCCCG, while the reverse primer is GACGCCGACATTAAGACGCAG with a target of 667 bp. Thermal cycler conditions with predenaturation parameters at 94°C for seven minutes, denaturation at 94°C for one minute, annealing at 59°C for 45 seconds, extension at 72°C for one minute 30 seconds, cycle repeated 35 times and extension final at 72°C for five minutes. Each primer is calibrated first to determine the appropriate annealing temperature and amplification using PCR. After that, the amplicons were visualized by electrophoresis using 2% agarose gel (Ramaditya et al. 2019).

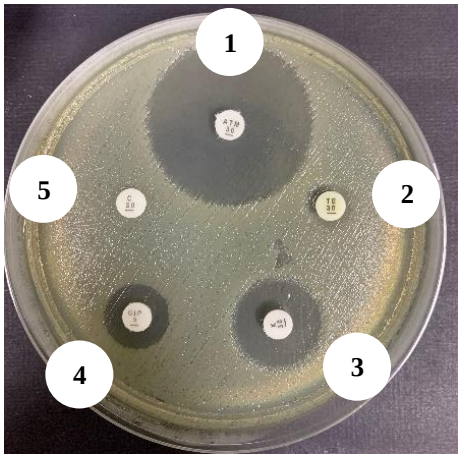

Figure 1. Multidrug Resistant (MDR) of *Escherichia coli*. Note: 1. Aztreonam; 2. Tetracycline; 3. Kanamycin; 4. Ciprofloxacin; 5. Chloramphenicol

RESULTS AND DISCUSSION

The results of the isolation and identification of *Escherichia coli* bacteria in this study obtained from 96 cloacal swabs of broiler chickens in traditional markets in Surabaya showed that 58/96 (60.4%) isolates were positive for *Escherichia coli*, and the results of *Escherichia coli* experiencing multidrug resistance were 25.8% (15/58), as shown on Table 1.

Samples were taken using enrichment media in the form of Buffered Peptone Water (BPW), which was then isolated on Eosin Methylene Blue Agar (EMBA) media. The macroscopic image of *Escherichia coli* growing on EMBA shows metallic green colonies characteristic of bacterial colonies that ferment lactose and a black dot in the colony's center.

The results of antibiotic resistance showed that the highest incidence occurred in tetracycline antibiotics at 56% (33/58), followed by antibiotic resistance to ciprofloxacin at 55% (32/58), aztreonam antibiotics at 29% (17/58), kanamycin antibiotics 20% (12/58), and chloramphenicol antibiotic resistance 18% (11/58), as shown in Table 2.

These results are based on the results of sensitivity tests which provide an interpretation (Table 3) of multidrug resistance in *Escherichia coli* isolates with resistance patterns ATM/TET/CIP/C; there are two samples (2/15; 13.3%), TET/K/CIP/ C is three samples (3/15; 20%), TET/CIP/C is three samples (3/15; 20%), TET/K/CIP is three samples (3/15; 20%), TET/K /C is two samples (2/15; 13.3%), ATM/TET/CIP is one (1/15; 6.67%), and the last resistance pattern is ATM/K/CIP one sample (1/15; 6.67%).

The results showed that six of the 15 *Escherichia coli* isolates rise to a positive band at 667 bp, so 40% of the *Escherichia coli* isolates had the iroN virulence gene in this study, as shown in Figure 2.

Table 1. Isolation and identification of *Escherichia coli*

Traditional market Surabaya	Sample size	*Escherichia coli*			
		Positive	Percentage (%)	Multidrug resistance	Percentage (%)
Dukuh Kupang (DK)	24	12	50%	3	25%
Keputran (PK)	24	14	58.3%	7	50%
Pucang (PP)	24	19	79.2%	4	21%
Pacar Keling (PC)	24	13	54.2%	1	7.69%
Total	96	58	60.4%	15	25.8%

Table 2. Profile of antibiotics resistant on *Escherichia coli* bacteria

Traditional market Surabaya	Sample size	ATM		TET		K		CIP		C	
		R	%	R	%	R	%	R	%	R	%
Dukuh Kupang (DK)	12	8	66%	4	33%	4	33%	5	41%	3	25%
Keputran (PK)	14	2	14%	9	64%	4	28%	9	64%	5	35%
Pucang (PP)	19	6	31%	10	52%	2	10%	15	78%	2	10%
Pacar Keling (PC)	13	1	7.6%	10	76%	2	15%	3	23%	1	7.6%
Total	58	17	29%	33	56%	12	20%	32	55%	11	18%

Note: ATM: Aztreonam, TET: Tetracycline, K: Kanamycin, CIP: Ciprofloxacin, C: Chloramphenicol

Table 3. Resistant pattern on multidrug-resistant *Escherichia coli*

Sample code	Resistant to antibiotic					Resistant pattern
	ATM	TET	K	CIP	C	
DK17	R	R	I	R	R	ATM/TET/CIP/C
PP1	R	R	I	R	R	ATM/TET/CIP/C
DK7	I	R	R	R	R	TET/K/CIP/C
PK20	S	R	R	R	R	TET/K/CIP/C
PK21	S	R	R	R	R	TET/K/CIP/C
PK1	S	R	S	R	R	TET/CIP/C
PK2	S	R	S	R	R	TET/CIP/C
PP20	S	R	S	R	R	TET/CIP/C
PP7	S	R	R	R	S	TET/K/CIP
PP16	S	R	R	R	S	TET/K/CIP
PC11	S	R	R	R	S	TET/K/CIP
DK23	S	R	R	I	R	TET/K/C
PK13	S	R	R	I	R	TET/K/C
PK3	R	R	S	R	S	ATM/TET/CIP
PK7	R	S	R	R	S	ATM/K/CIP

Note: ATM: Aztreonam, TET: Tetracycline, K: Kanamycin, CIP: Ciprofloxacin, C: Chloramphenicol, R: Resistant, S: Sensitive

Discussion

The use of antibiotics in poultry infected with bacterial diseases, such as colibacillosis, is necessary to improve animal welfare and reduce economic losses due to colibacillosis (Tohmaz et al. 2022). However, unwise use can cause antibiotic resistance (Wall 2019; Ansharieta et al. 2021). Antibiotic resistance can be obtained from mutase, gene transfer via conjugation or transformation, transposons, integrons, and bacteriophages (Hardiati et al. 2021a). According to (World Health Organization 2017), *Escherichia coli* is the main bacteria that can cause the spread of antibiotic resistance.

This study showed a resistance rate to tetracycline antibiotics of 56% (33/58) with a high resistance level. Previous research stated that *Escherichia coli* isolates were highly resistant to 100% tetracycline in Cianjur (Hardiati et al. 2021a); tetracycline resistance was 97% (Niasono et al. 2019). The results of this study are lower than in Bangladesh, namely tetracycline resistance of 100% in layer chickens (Ievy et al. 2022) and higher than in Italy of 43% in poultry (Sgariglia et al. 2019). Tetracycline antibiotics are often used as growth promoters in livestock worldwide (Azizah et al. 2022).

The incidence of *Escherichia coli* resistance to ciprofloxacin was 55% (32/58). Results of antibiotic resistance in broiler chicken strains of Avian Pathogenic *Escherichia coli* (APEC), respectively; 87.5% in Algeria (Aberkane et al. 2023); 97% in healthy chickens and 100% in sick chickens in Qatar (Johar et al. 2021); broiler chicken carcasses 86% in Iran (Bakhshi et al. 2017); broiler chickens by 72% in Pakistan (Azam et al. 2019) and in the broiler chicken environment by 45.9% in West Java (Niasono et al. 2019). Ciprofloxacin is generally broad-spectrum and is often used to treat respiratory, urinary, and digestive tract infections (Tchesnokova et al. 2023).

This study also showed that *Escherichia coli* was resistant to the antibiotic aztreonam 29% (17/58). Aztreonam is a monobactam antibiotic that works by binding to gram-negative protein 3 (PBP-3), causing lysis and death of bacteria (Rahmahani et al. 2020). The mechanism of aztreonam resistance is caused by bacteria that produce the Extended Spectrum Beta Lactamase (ESBL) enzyme, which can inhibit the antibiotics penicillin, cephalosporins, and aztreonam (Saeed et al. 2023). Chickens are a source of ESBL, which can be transmitted to humans through direct contact, the food chain, or environmental contamination (Aberkane et al. 2023). *Escherichia coli* experienced resistance to the antibiotic kanamycin 20% (12/58) in this study. This result is quite low compared to previous research on APEC broiler chicken strains in Egypt, namely 69% (Awad et al. 2016) and 80.19% resistance to kanamycin in broiler chickens in China (Xu et al. 2019).

Previous studies showed that *Escherichia coli* was resistant to the antibiotic chloramphenicol, respectively, at 80.19%, 30%, and 27.5% in broiler chickens in China, Egypt, and Algeria (Aberkane et al. 2023; Xu et al. 2019; Younis et al. 2017). This result is lower than the study, which showed *Escherichia coli* resistance to the antibiotic chloramphenicol was 18% (11/58). This result is higher than that for chickens in the Bangladeshi poultry industry at 8.9% (Hasan et al. 2011). Chloramphenicol has been banned in animal husbandry, low level resistance may be due to previous resistance or illegal use (Hardiati et al. 2021a).

Resistance patterns show diversity due to various antibiotics, geographical differences, and different poultry production systems, causing differences in resistance patterns (Hardiati et al. 2021a). The multidrug resistance (MDR) incidence in *Escherichia coli* isolates was 25.8% (15/58) obtained from broiler chicken cloaca swabs at Surabaya traditional markets. This result shows that the higher MDR in Cianjur chickens is 90% (Hardiati et al. 2021b), and Blitar is 75% (Wibisono et al. 2020). MDR results on Avian Pathogenic *Escherichia coli* isolates have less reported in Indonesia. MDR results in Avian Pathogenic *Escherichia coli* (APEC) were 22% (Subedi et al. 2018) and in Brazil; 52.2% in Algeria (Aberkane et al. 2023). The incidence of multidrug resistance is increasing throughout the world due to the spread of genes located on genetic elements in the form of plasmids and the combination of genes with resistance genes, which produce bacteria resistant to all classes of antibiotics (Allocati et al. 2013; Hardiati et al. 2021b).

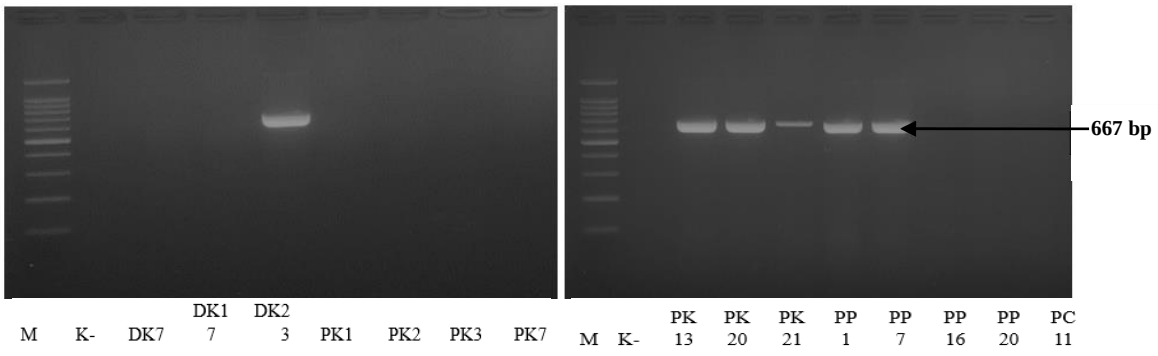

Figure 2. iroN gene on avian fecal *Escherichia coli* positive result on 667 bp. Sample codes DK23, PK13, PK20, PK21, PP1, and PP7 have iroN gene, while the others have negative results. Note: M: marker; K-: negative control; DK7 – PC 11: samples code

The incidence of colibacillosis in poultry has increased, causing the use of antibiotics for disease treatment and prevention, and one of the impacts of poultry farming is an increase in cases of Multidrug Resistance (MDR) (Wibisono et al. 2022; Bakhshi et al. 2017). The MDR of *E. coli* is a threat to global public health that requires action in all sectors of government and society. Uncontrolled use of antibiotics can cause MDR in laying hen farms (Wibisono et al. 2021) and broiler chickens (Wibisono et al. 2020). The high use of antibiotics without a doctor's prescription is caused by farmers' perception that its use has no detrimental effects and is a low-cost effort to prevent disease (Wibisono et al. 2021). However, bacterial resistance is a growing problem worldwide, and the World Health Organization's published statements regarding the importance of assessment factors associated with this problem and strategies to overcome it, controlling the incidence of MDR. The problem of MDR originating from poultry in one country will now become a problem for all countries (WHO 2017).

Foodborne illnesses from poultry impact animal and human health, including zoonotic transmission found in food of animal origin (Azam et al. 2019; Effendi et al. 2021). The presence of MDR from foodborne bacteria has caused the failure to treat gastrointestinal infections in humans. Foodborne bacteria exhibiting MDR properties can be transmitted to humans through the food chain or direct contact (Rahmahani et al. 2020; Effendi et al. 2021). Because there is a relationship between antibiotic resistance of foodborne bacteria and the occurrence of antibiotic resistance in humans, their use of antibiotics in the poultry industry must be controlled (Hu et al. 2022; Jeong et al. 2021).

Infectious diseases can attack broiler chickens, one of which is caused by Avian Pathogenic *Escherichia coli* (APEC) and is considered the main cause of health problems in poultry farms throughout the world (Dhaouadi et al. 2020; Luhung et al. 2017). APEC infection causes systemic symptoms, such as airsacculitis, pericarditis, perihepatitis, septicemia, enteritis, granuloma, sinusitis, omphalitis, peritonitis, and swollen head syndrome in poultry (Azam et al. 2019; Wang et al. 2022). Several genes in the plasmid cause virulence factors in APEC strains, such as cvaC, tsh, sitA, iutA, ompT, etsABCD, eitABC, and iroN. Pathogenic isolates have genes with a high frequency of around 85% compared to commensal bacteria, which is around 25% (Gunawan et al. 2020).

The PCR showed that six isolates (40%) had the iroN virulence gene. This result is lower than the reported incidence of APEC which is influenced by various iroN virulence genes of 100% in Bangladesh (Hossain et al. 2021), 92% in China (Subedi et al. 2018), 97% in Qatar, Korea 100% (Jeong et al. 2021) another study in Indonesia on APEC on iron genes showed 100% (Ramaditya et al. 2019), while in broiler chickens, it showed 100% (Gunawan et al. 2020). The iroN gene is commonly found in APEC because the iroN gene has siderophores (aerobactin, salmochelin, yersiniabactin), which are secondary metabolites that absorb iron to increase bacterial growth and development. The ability of the iroN gene to enter blood serum is very important because *Escherichia coli* can cause sepsis and infections in various organs that lack iron (Kathayat et al. 2021; Su et al. 2016). Even though PCR has found the iron gene, it is caused by E. coli isolated from feces; it is more correctly called Avian Fecal *Escherichia coli* (AFEC).

In conclusion, based on the results of antibiotic resistance research on cloacal swabs of broiler chickens in traditional markets in Surabaya, it showed that *Escherichia coli* was resistant to the antibiotic tetracycline at 56%, ciprofloxacin at 55%, the antibiotic aztreonam at 29%, kanamycin at 20% and chloramphenicol at 18%. The incidence of Multidrug Resistance (MDR) in *Escherichia coli* isolates was 25.8%, and the Avian Fecal *Escherichia coli* (AFEC) virulence gene, namely iroN, was 40%. In addition, APECs that have iroN gene pose the potential to affect public health.

ACKNOWLEDGEMENTS

This study was partly supported by the International Research Collaboration Top Over #500 funding from *Lembaga Penelitian dan Pengabdian Masyarakat*, Universitas Airlangga, Indonesia, in the fiscal year 2023.

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